12-6-2013

The Effects of Poor Maternal Nutrition on Muscle Development in Lambs

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B.Tech., Dr. D.Y. Patil University, 2011

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

at the

University of Connecticut

2013

APPROVAL PAGE

Master of Science Thesis

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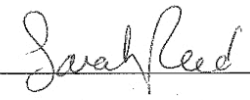
Presented by Joseline Subala Raja, B.Tech.

Major Advisor 

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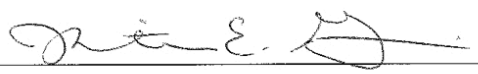
Associate Advisor 

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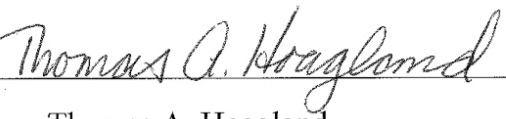
Associate Advisor 

Thomas A. Hoagland

University of Connecticut

2013

Master of Science Thesis

DEDICATION

I would like to dedicate this thesis to my parents, Anthony and Joyce Raja, and my grandparents Jesu and Josephine Rajiah.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my major advisor, Dr. Sarah Reed, who was a mentor, a friend and a great guide throughout my Masters' journey. My sincerest gratitude also extends to my associate advisors, Dr. Kristen Govoni and Dr. Thomas Hoagland for their guidance.

I would also like to acknowledge the support and help of my labmates Elisabeth Leahy and Bethany Sullivan, and all the past and present undergraduate members of the Reed Lab, Govoni Lab and Lee Lab

The love and warmth of all the Animal Science graduate students, the faculty and the staff were also instrumental in helping me cruise through these two years smoothly.

Finally, I want to thank all my friends and relatives back in India and in the U.S. for their guidance, and moral support.

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ABBREVIATIONS

ActR	Activin Receptor
bHLH	basic Helix Loop Helix
BMP-7	Bone Morphogenetic Protein 7
CCL2	Chemokine (C-C) motif ligand -2
CON	Control
CSA	Cross Sectional Area
FGF	Fibroblast Growth Factor
FLRG	Follistatin related gene
FOXO	Forkhead box O
Fst	Follistatin
GASP	G-Protein Associated Sorting Protein
GDF-8	Growth and Differentiation Factor 8
GH	Growth Hormone
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
IGF-1	Insulin-like Growth Factor 1
IGF-1R	Insulin-like Growth Factor 1 receptor
IKK	Inhibitor of κ kinase
IL	Interleukin
IRS-1	Insulin Receptor Substrate 1
IUGR	Intra-uterine Growth Retardation
JNK	c-Jun N-terminal Kinase
kDa	Kilodalton

Lbx	Ladybird homeobox
LGA	Large for Gestational-Age
MAPK	Mitogen Activated Protein Kinase
MEF2	Muscle-Specific Enhancer Factor 2
MRF	Myogenic Regulatory Factor
mTOR	Mammalian Target of Rapamycin
MyHC	Myosin Heavy Chain
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NRC	National Research Council
O.C.T	Optimal Cutting Temperature compound
OVER	Overfed
pAkt	Phosphorylated Akt
Pax	Paired box
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGC-1α	Proliferator-activated receptor γ coactivator 1α
PKR	Protein kinase R
PPARγ	Peroxisome proliferator-activated receptor γ
PVDF	Polyvinylidene fluoride
RES	Restricted
ROS	Reactive Oxygen Species
RT	Room Temperature
SEM	Standard Error of Mean
Ser	Serine
SGA	Small for Gestational-Age

Shh	Sonic Hedgehog
SOCS	Suppressor of Cytokine Signaling
Tcf/Lef	T-Cell factor/Lymphoid enhancing factor
TGFβ	Transforming Growth Factor β
Thr	Threonine
TLR	Toll-like Receptor
TNFα	Tumor Necrosis Factor α
WGA	Wheat Germ Agglutinin
Wnt	Wingless and Int

Review of Literature

Introduction

Skeletal muscle is a specialized heterogeneous tissue with individual muscle fibers varying in size, metabolic and contractile properties. It is composed of multinucleated muscle fibers and has a striated appearance due to the organization of the contractile units. Skeletal muscles make up a large portion of the body and play important roles in locomotion, maintaining posture, glucose and fatty acid metabolism among others. Thus, it is of great importance to understand the process of muscle formation and the various factors that affect myogenesis.

Prenatal Myogenesis

Skeletal muscles in vertebrates are mesodermal in origin (Maroto et al., 1997; Fan et al., 2012). They are formed from specialized paired segmental units of paraxial mesoderm known as somites that are formed on either side of the neural tube and notochord. Somites differentiate into sclerotome, myotome, and dermatome, which form the cartilage, muscle and skin respectively (Stern et al., 1988). The differentiation of the epithelial cells in the somites to cells of the myogenic lineage is prompted by signals from the surrounding tissues (Buckingham, 2001). Two of the earliest positive effectors of myogenesis to be identified are Wingless and Int (Wnt), synthesized by the dorsal neural tube and ectoderm, and Sonic Hedgehog (Shh), produced by the notochord and floor plate of the neural tube (Buckingham, 2001). Epithelial-mesenchymal transformation results in the formation of a large number of multipotent mesenchymal stem cells that are common precursors of myocytes, adipocytes and chondrocytes (Grigoriadis et al., 1988). An important protein, β-catenin, modulates the dermomyotome formation and the delamination of the limb myogenic progenitors (Linker et al., 2003, Schmidt et al., 2004, Hutchenson et al.,

2009). Activation of the Wnt/β-catenin complex stabilizes β-catenin protein and causes it to translocate to the nucleus, where it binds the T-cell factor/Lymphoid enhancing factor (Tcf/Lef) family of transcription factors increasing the expression of the myogenic regulatory factors (Miller et al., 1999).

Myogenic precursor cells that lie dorso-medially in the somite give rise to the axial muscles, whereas those that lie in the ventral-lateral part of the somite form the muscles of the limbs (Ordahl and Douarin, 1992). On receiving positional cues from the mesenchymal cells in the limb bud (Christ et al., 1995), the muscle progenitor cells delaminate from the somite, and migrate into the limb field (Buckingham et al., 2003). The delamination and the movement of these precursor cells is controlled by a tyrosine kinase receptor, c-met and its ligand, Hepatocyte growth factor/Scatter factor (HGF/SF, Buckingham et al., 2003). The mutation of either of these genes leads to impaired limb formation (Dietrich, 1999) as a result of the improper colonization of the limb bud by the myogenic precursor cells (Bladt et al., 1995). Ladybird homeobox 1 (Lbx1) also plays a pivotal role in the migration of cells from the somite into the limb field, while maintaining the myogenic precursor cells in an undifferentiated state. A mutation in the Lbx1 gene leads to premature differentiation of the muscle precursor cells during migration (Gross et al., 2000).

Primary Myogenesis

The process of primary myogenesis occurs during the embryonic stage of growth. In sheep, primary myogenesis begins around d 32 of embryonic age, and is most active around d 38, when about 60% of primary myofibers are formed following which secondary myogenesis commences (Wilson et al., 1992). The development of the dermomyotome from the somite is

driven by the secretion of Wnt from the surrounding structures (Fan et al., 1997). The neural tube expresses Wnt1 and Wnt3a, while Wnt4 and Wnt6 are expressed in the dorsal surface ectoderm (Parr et al., 1993). Wnts regulate myogenesis by controlling the activity of myogenic regulatory factors (MRFs) in a concentration-dependent manner (Zecca et al., 1996, Stringini et al., 2000). MRFs are basic helix-loop-helix transcription factors that specifically regulate the process of myogenesis. The process of myogenesis is also very strictly regulated by a group of paired box transcription factors: Pax3 and Pax7 (Messina et al., 2009). The determination, proliferation, and migration of $Pax3^{+}/Pax7^{+}$ cells are mediated by β-catenin (Hutchenson et al., 2009). Post migration, the embryonic cells do not express β-catenin. However, it has been observed that in the absence of β-catenin, the total number of $Pax7^{+}$ fetal myoblasts and the myofibers formed is decreased, which indicates the probable function of β-catenin in regulating proliferation of myoblasts (Messina et al., 2009).

The precursor cells reach the limb and begin expressing the MRFs, MyoD and Myf5, both of which are crucial for proper myogenesis (Tajbakhsh and Buckingham, 1994). The muscle progenitor cells rapidly proliferate before and during the expression of these two early MRFs. MyoD, Myf5 and Pax3 regulate the cell cycle, maintaining the cells in a proliferative state (Delfini et al., 2000). The cells then withdraw from the cell cycle, transform from a rounded shape to a more elongated morphology and start forming immature myofibrils, at which time they are identified as myoblasts (Fürst et al., 1989). Myoblasts gradually acquire a spindle shape and the myofibrils fill the cytoplasm. With the progression of development, the mono-nucleated myoblasts fuse to form syncytia with the nuclei aligned at the center. As they mature, the syncytia are called myotubes and exhibit the myofibrillar arrangement similar to the adult muscle (Fürst et al., 1989). The final maturation is indicated by the position of the nuclei, which

are pushed to the periphery of the myotubes, and a significant increase in the size of the muscle fibers. Primary fibers are slow twitch fibers and are very few in number (Yan et al., 2012). Primary myofibers are densely innervated prior to the beginning of the second wave of myogenesis and form a scaffold for the formation of secondary myotubes (Ross et al., 1987). These myofibers on an average constitute about 2% of the total number of muscle fibers in a adult muscle (Wilson et al., 1992).

Secondary Myogenesis

Secondary myogenesis coincides with the formation of adipocytes and chondrocytes from mesenchymal stem cells. The secondary myotubes appear as short cells lying beneath the basal lamina of the primary myotubes. The late-migrating population of myoblasts has been hypothesized to be precursors of the secondary myotubes (Duxson, 1992). Mononucleated myoblasts fuse together near the endplates of the primary myofibers and continue extending by the fusion of multiple subsequent myoblasts using primary myofibers as templates and aligning longitudinally to their surface. The secondary myofibers secure themselves on the surface of the primary myofibers by inserting protrusions and interdigitating with them (Wilson et al., 1992). In mice, clusters of secondary fibers are initially ensheathed in a single basal lamina, and after birth these individual fibers separate out and are each enclosed in separate basal laminas (Rosen et al., 1992). In sheep the formation of secondary myotube is terminated when the next tendon is reached, as the primary myotube extends from one tendon to the next. Unlike the primary myotube that connects two tendons in a single fiber, there are multiple secondary myotubes formed along the longitudinal plane of the primary myofiber that lie lateral to each other (Wilson et al., 1992). On reaching the next tendon all the secondary myofibers formed from the same

primary myofiber detach from their scaffold. These immature secondary myofibers have a smaller diameter compared with the primary myofibers. Secondary myogenesis continues until d76 in sheep (Wilson et al., 1992).

Tertiary myogenesis

Studies by Kell and Zacks (1969), and Ross et al. (1987), suggest a biphasic pattern of muscle fiber development. This phenomenon could not explain the observation that the ratio of secondary to primary myofibers is 70:1, which means that one primary myofiber serves as a scaffold for 70 secondary myofibers. Wilson and colleagues in 1992 observed that by d76 in sheep there were fibers of large, intermediate and small diameters in the tibialis cranialis muscle. The variation in the sizes of the three kinds of fibers and the observation that they were not apposed to each other led them to conclude that there are three waves of myogenesis. They stated that the fibers with the largest, intermediate and smallest diameters were the primary, secondary and tertiary myofibers respectively, which they could deduce on the basis of the maturity of the fibers at d76. Following the three phases of myogenesis prenatally, the muscle fibers form rosettes, which are organized into fascicles. Individual fibers increase in size by hypertrophy till birth after the formation of fascicles (Maier et al., 1992).

Types of muscle fibers

Muscle is a heterogeneous tissue, with each muscle consisting of more than one type of fiber. This enables one muscle to assist in multiple functions. The contraction of muscle fibers is mediated by free Ca^{2+} ions in the muscle cells, which associates with troponin, a regulatory

protein of the actin filament. This binding permits the interaction between actin and myosin head, resulting in contraction of the muscle fiber. When the Ca^{2+} ion concentration in the muscle fibers drops, contractile activity declines. In the early nineteenth century researchers broadly classified muscle fibers of the skeletal muscle on the basis of color as red or white, or on the basis of their contractile properties as fast or slow twitch fibers (Cooper and Eccles, 1930). Current classification is based on the glycolytic or oxidative properties of the muscle fibers and the isoform of myosin heavy chain (MyHC) present in the fibers (Schiaffino and Reggiani, 2011b). The two main types of muscle fibers are Type I and Type II.

Type I

Type I fibers are slow twitch fibers. They are red in color due to the presence of a large amount of myoglobin and are highly infused with capillaries. These fibers utilize triglycerides and carbohydrate for contraction and have the highest oxidative and the least glycolytic properties among all the muscle fibers. They split ATP at a very slow rate, hence the slow contraction velocity (Wang et al., 2004). Type I fibers are usually found in abundance in the postural muscles and are best used in activities that require endurance. These myofibers are resistant to fatigue partly due to the abundance of mitochondria and the highly active oxidative enzymes (Schwerzmann et al., 1989).

Type II

Type II fibers are fast twitch fibers and can be divided into three subtypes: Type IIa, IIb and IIx. Type IIa fibers are oxidative, fast twitch fibers. These are also red due to an abundance

of myoglobin. Type IIa fibers utilize carbohydrates (mostly glycogen) and triglycerides for contraction, but unlike the Type I fibers, the splitting of ATP is rapid, hence the fast rate of contraction (Greenhaff et al., 1993). These fibers are moderately resistant to fatigue and are found in muscles that need to contract and relax at a fast pace. Type IIb fibers are glycolytic, using glycogen as fuel for contractions. They have significantly fewer capillaries and myoglobin content and are white in color. These fibers have the fastest speed of contraction and are quickly fatigued. Type IIx fibers have properties intermediate to Type IIa and Type IIb fibers. These fibers are fast with an intermediate resistance to fatigue. Type IIx fibers are found in abundance in the diaphragm (Schiaffino et al., 1989).

Primary myotubes generally express the slow myosin heavy chain isoform while secondary myotubes express the fast myosin heavy chain isoform to mature into Type II fibers (Pin et al., 2002). The determination of fiber types occurs at the myoblast stage prior to the fusion of myoblasts to form myofibers. Intrinsic factors like the myoblast lineage and extrinsic factors like innervation play primary roles in the determination of the fiber type of a muscle fiber (DiMario and Stockdale, 1997). However, there can be switching from one fiber type to another postnatally due to various factors like mutation in an essential gene namely peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (Handschin et al., 2007), natural switch from embryonic to adult phenotypes (Whalen et al., 1984; Agbulut et al., 2003) or a need for the body to regulate insulin better (Ryder et al., 2003).

Postnatal Myogenesis

Among all the time periods postnatally, the neonatal phase has the highest growth rate, to which the skeletal muscles contribute the majority of the increase in body mass. The increase in muscle mass postnatally in mammals like sheep, cattle and humans is through hypertrophy which is an increase in the size of muscle fibers leading to a net gain in the mass of the whole muscle. There is no production of new fibers after birth (Wigmore and Stickland, 1982). The increase in muscle mass is attributed primarily to protein accretion. The protein synthesis of the myofibrillar proteins supersedes the degradation of these proteins in the neonatal period, hence the increase in muscle mass. As the animal ages, protein synthesis slows, until the rate of protein synthesis and protein degradation strike equilibrium, during which there is no growth in the muscle fibers (Davis et al, 1989).

Protein accretion in the muscle fibers is accompanied by an increase in the myonuclear content of the fiber, which is brought about by satellite cells in the muscle. Satellite cells are the primary stem cells of the muscle that lie between the basal lamina and the sarcolemma of the muscle fibers. These cells reside in a quiescent state but can be stimulated to enter the cell cycle (Siegel et al., 2011). Following activation, satellite cells proliferate and a portion of these cells fuse into the preexisting muscle fibers while the rest of the cells replenish the repository of satellite cells. This reservoir of satellite cells decreases with age in rats (Allbrook et al., 1971) and mice (Day et al., 2010), but there are no such studies in sheep. The decline in the satellite cell population can be attributed partly to an increased postnatal Wnt expression, which promotes the myogenic precursor cells to transform to the fibrogenic lineage (Knittel et al., 1999; Willis et al., 2006; Brack et al., 2007). Satellite cells also play a major role in the regeneration of the muscle fibers postnatally in the event of injury to the muscle fibers. The activation of satellite

cells follows a series of steps. The c-met-hepatocyte growth factor (HGF) pathway, Notch, and some members of the fibroblast growth factor (FGF) are instrumental in stimulating satellite cells to enter the cell cycle (Shefer et al., 2006). Proliferating satellite cells increase the expression of Pax7, and decrease the expression of CD34 (a marker of the hematopoietic stem cells (Beauchamp et al., 2000). The expression of Pax7 stimulates the ordered expression of myogenic regulatory factors required for successful myogenesis.

Myogenic Regulatory Factors

A family of closely related transcription factors, the myogenic regulatory factors (MRFs) tightly regulate the process of myogenesis (Rescan, 2001). The four MRFs are MyoD, Myf5, myogenin and MRF4. MyoD and Myf5 are expressed in the proliferating myoblasts (Emerson, 1990) and play a pivotal role in the specification of progenitor cells (Pownall et al., 2002). Myogenin and MRF4 are expressed later in the process of myogenesis (Rhodes and Konieczny, 1989) when myoblasts are differentiating and fusing (Edmundson and Olson, 1989; Miner and Wold, 1990).

Myf5 and MyoD

Myf5 is a basic helix loop helix (bHLH) transcription factor necessary for the differentiation of myoblasts. It is the earliest of the MRFs to be expressed by the precursors of myoblasts. Its expression is observed first in the dorsomedial lip of the dermomyotome on E 8 in mice (Ott et al., 1991). Myf5 plays a vital role in the differentiation of the myogenic precursors, but by itself is not sufficient to orchestrate proper muscle development (Kablar et al., 1997).

MyoD belongs to a large family of DNA-binding proteins containing a bHLH domain. It

mediates the formation of muscle by binding to the regions of myogenic genes that promote the transcription of genes like myocyte enhancer factor-2 (Mef-2) and p38 mitogen activated protein kinase (MAPK) (Baldwin and Burden, 1989, Penn et al., 2004). MyoD aids in the generation of myogenic cells even without extracellular stimulation like those from adhesion molecules, collagen fibers, proteoglycans, and other signaling molecules. MyoD plays a significant role in postnatal myogenesis as well. MyoD mutant mice have a defect in satellite cell proliferation and regeneration in these mice was delayed (Cornelison et al., 2000), although they do not exhibit any major muscle abnormalities (Rudnicki et al., 1993).

Myf5 and MyoD have compensatory actions. Double knock out mice for MyoD and Myf5 survive, but cannot form skeletal muscles (Kaul et al., 1992; Rudnicki et al., 1992). Owing to this defect, the respiratory system in these mutant embryos is also not formed, and hence they do not live long (Braun et al., 1992). Some embryos that do survive are immobile and die shortly after birth. The lack of skeletal muscle is compensated for by the accumulation of loose connective tissue and adipose tissue (Rudnicki et al., 1993). The expression of Myf5 usually declines with the progression of myoblast differentiation. However, in MyoD mutant (-/-) mice, the expression of Myf5 increases by three to four fold (Rudnicki et al., 1992) to compensate for the loss of MyoD which aids in progenitor specification, but the formation of limb muscles in these animals is delayed significantly (Kablar et al., 1997). The differentiation of limb progenitors in Myf5 mutant mice (-/-) occurs normally (Kablar et al., 1997), however, the MyoD expression in the progenitor cells is slightly delayed (Pownall et al., 2002), suggesting that Myf5 controls the timing of MyoD activation. Apart from specification, MyoD and Myf5 have also been shown to play a major role in proliferation as well as the initiation of differentiation of the progenitor cells (Montarras, 2000; Pownall et al., 2002).

Myogenin

Like the other MRFs, myogenin is a bHLH protein, which functions as a transcription factor to promote myogenesis. It acts downstream of MyoD and Myf5 and is a marker of terminal differentiation in the cells. MyoD increases transcription of myogenin, which can increase its own transcription as well as that of MyoD. Muscle-Specific Enhancer Factor 2 (MEF2) also induces the expression of myogenin (Edmondson et al., 1992). Following the expression of myogenin in the myogenic precursor cells, a series of highly ordered processes occur including the induction of cyclin dependent kinase p21, the withdrawal of these cells from the cell cycle, development of the contractile apparatus of the muscle cells and the fusion of the myoblasts to form multinucleated myotubes (Andrés and Walsh, 1996).

MRF4

Myogenic Regulatory Factor 4 (MRF4) is the last of the MRFs to be expressed in developing muscles and plays a role in the maturation of myofibers. Myogenin and MRF4 regulate the expression of genes coding for muscle contractile proteins like alpha actin and troponin I reporter genes (Mak et al., 1992; Pownall et al., 2002). Mice with a myogenin/MRF4 null mutation do not form functional myocytes, although they do form progenitor cells that express MyoD (Nabeshima et al., 1993; Venuti et al., 1993; Pownall et al., 2002), supporting the role of these factors in progenitor cell differentiation. In some studies (Rawls et al., 1998), these animals formed a few residual fibers, which suggests that neither of these genes is involved in the activation of differentiation and that MyoD has overlapping functions with MRF4 and can initiate the process of differentiation. Triple mutants of MyoD (-/-), Myogenin (-/-), and MRF4 (-

/-) fail to produce differentiated muscle fibers even though they have the normal number of progenitor cells (Valdez and Richardson, 2000).

Regulation of Myogenesis

Apart from MRFs, myogenesis is also governed by many extracellular factors including myostatin, follistatin and growth factors. The timely expression of these factors in the appropriate physiological levels is essential for myogenesis to proceed normally.

Myostatin

Myostatin, also known as Growth and Differentiation Factor 8 (GDF-8) is an important member of the Transforming Growth Factor β (TGF β) family of proteins (McPherron et al., 1997). The 52 kDa precursor form of the protein is proteolytically cleaved to release the active isoform of the protein. The propeptide region of the protein plays an important role in correct folding, regulating the activation of the C-terminal region of the protein. The expression of myostatin is observed in the myotome of the developing embryo and continues through embryogenesis and in adulthood (Lee, 2004). Myostatin has an inhibitory effect on myogenesis (Grobert et al., 1997; McPherron et al., 1997). Activated myostatin binds to the serine/threonine kinase receptors activin receptors (ActR) IIA and IIB and inhibits myoblast proliferation (Thomas et al., 2000) as well as differentiation (Joulia et al., 2003). Myostatin also inhibits protein synthesis (Taylor et al., 2001).

A mutation in the myostatin gene results in increased muscle mass and a decrease in adiposity, giving rise to a condition known as ‘double muscling’. This phenotype is observed in

Belgian blue cattle that have an 11 bp mutation in the myostatin gene (Grobert et al., 1997; Lin et al., 2002). The increase in muscle mass is a combined effect of hyperplasia (an increase in the number of muscle fibers) and hypertrophy (an increase in the size of muscle fibers; Lee, 2004). Follistatin (Nakamura, 1990), follistatin related gene (FLRG; Hill et al., 2002) and G-protein coupled receptor associated sorting protein (GASP; Hill et al., 2003) regulate the activity of myostatin by binding to myostatin. Postnatally, blocking myostatin activity improves the physical function and performance of muscle and has been suggested as a therapeutic option in attenuation of myogenic disorders like muscular dystrophy. The replacement of muscle fibers by fibrotic and adipose tissue was diminished considerably (Wagner et al., 2002, LeBrasseur et al., 2009).

Follistatin

Follistatin is a 38 kDa secreted glycoprotein that antagonizes the effect of myostatin (Michel et al., 1993). It prevents the binding of myostatin to its receptor by binding to myostatin protein, alleviating the muscle loss brought about by myostatin. Crystallographic study of the protein shows the presence of an N terminal domain and three cysteine-rich domains, FS I, FS II and FS III, each having distinct functions (Thompson et al., 2005; Harrington et al., 2006). Blocking myostatin with the FS I domain in mice has therapeutic potential for muscular dystrophy by increasing the muscle size and recovering muscle strength (Nakatani et al., 2008). The expression of follistatin in the somites precedes that of myostatin (Amthor et al., 1996), suggesting other roles of follistatin in muscle development. It is present in the sites of probable muscle development and possibly negates the effect of molecules that hinder the development of muscle tissue (Matzuk et al., 1995). Follistatin also binds to bone morphogenetic protein-7

(BMP-7) and converts the muscle growth inhibiting effect of the protein to a stimulatory effect (Amthor et al., 2002). Mice mutant in follistatin have several defects including decreased growth rate and muscle mass, taut skin, and poor bone development. Mice with a homozygous mutation for follistatin were born with reduced muscle mass and died shortly after birth (Matzuk et al., 1994). In $Fst^{+/-}$ mutant mice, the muscle mass was reduced by 15 to 20 %, and the muscle fibers were much smaller than those from control mice. There was no significant effect on the number of muscle fibers in these mice (Lee et al., 2010).

Akt

Akt, also known as protein kinase B, is a serine/threonine kinase that regulates many cellular processes including cell proliferation (Shin et al., 2002), apoptosis and glucose metabolism (Jeffrey et al., 2003). In the muscle, Akt mediates growth, which mediates Insulin-like growth factor 1 (IGF-1) signaling. IGF-1 plays an important role in muscle homeostasis (Alessi and Cohen, 1998; Sciaffino and Mammucari, 2011) by binding of IGF-1 to its receptor (IGF-1R) and initiating a cascade of phosphorylation steps and activation of intermediates, followed by the subsequent phosphorylation of Akt (pAkt). pAkt stimulates protein synthesis by indirectly activating the serine/threonine kinase, mammalian target of rapamycin (mTOR; Kitamura et al., 1998). It also inhibits protein degradation by phosphorylating and inactivating the FOXO proteins, which negatively regulate processes like cell growth and proliferation. (Sciaffino and Mammucari, 2011). Another well-studied activator of Akt is the pleiotropic cytokine Transforming Growth Factor (TGFβ), which promotes myoblast differentiation by phosphorylation and hence the activation of Akt (Kulkarni et al., 2010; Suwanabol et al., 2012).

Disruption in the *Akt1* gene causes retardation of growth, and a deletion of *Akt2* gene alters glucose metabolism, but not growth (Chen et al., 2001; Cho et al., 2001). Mice with knockout of Akt1 and Akt2 genes exhibited muscular dystrophy, improper adipogenesis, bone formation and skin formation, accompanied with dwarfism (Peng et al., 2003). When Akt expression in these animals was induced between 1-3 weeks, an improvement in muscle hypertrophy was observed together with increased strength of the muscle. However, there was no incorporation of satellite cells in muscles of Akt double knockout mice with brief Akt intervention (Blaauw et al., 2008; 2009).

Growth Factors

Growth factors are important regulators of muscle growth and can have dramatic effects on myoblast proliferation, differentiation and muscle growth. IGF-1 is a hormone that is structurally similar to insulin, which is synthesized in the liver and other target organs by the stimulation of growth hormone. IGF-1 promotes protein synthesis in the muscle via activation of the PI3K/mTOR pathway (Schiaffino and Mammucari, 2011a) and inhibits protein degradation by blocking the activity of FOXO proteins (Guertin et al., 2006). Growth hormone (GH) also regulates myogenesis by increasing muscle strength and decreasing the adiposity of the muscle. Mice that are deficient in the GH receptor have decreased muscle mass and improper specification and growth of myofibers (Baum et al., 1996). GH stimulates the secretion of many proteins including IGF-1. It promotes the proliferation and fusion of myoblast via the activation of IGF-1 (Mavalli et al., 2010).

Fibroblast growth factor (FGF) is a heparin-binding regulator of various processes in

embryonic development including angiogenesis and muscle development. FGF2 protein expression is observed in the mesoderm and apical ridge of the ectoderm, and plays a vital role in the outgrowth of the limbs (Olwin et al., 1994). The FGF family of proteins regulates the balance between proliferation and differentiation of myoblasts by causing the activation of MyoD. Inhibition of FGFR4 (a receptor for FGF) leads to a significant loss of limb muscles (Marics et al., 2002).

Maternal Nutrition and Fetal Development

Many factors can affect myogenesis prenatally and postnatally including protein metabolism, micro damage to the muscle fibers, failure in the myogenic contractile machinery, environmental stresses and maternal nutrition. Maternal nutrition during gestation affects myogenesis prenatally and can affect muscle development postnatally as well. Poor maternal nutrition refers to both under and over nutrition as well as deficiencies in specific nutrients like proteins, vitamins or minerals.

The 'thrifty phenotype' hypothesis or Barker's theory was put forward by Dr. David Barker in 1992, who proposed that poor nutrient availability during fetal growth alters the physiology and metabolism of the fetus, predisposing it to chronic metabolic defects later in life (Barker 1992; Hales and Barker, 2001). Slower growth rate at the fetal age increases stress responses in the body and makes the body vulnerable to poor health conditions later in life. Low birth weight, low ponderal index (birth weight/height^3) and large for gestational-age (LGA) conditions are a few of the factors resulting primarily by poor maternal nutrition that favor the development of metabolic disorders in adolescence (Eriksson et al., 2001; Bonet et al., 2005).

Poor lifestyle and insufficient education about the effects of poor nutrition both contribute to a rising number of infants born with intra-uterine growth retardation (IUGR) worldwide (Kramer et al., 1990; Neel and Alvarez, 1991). Research performed in the 1990's associated IUGR with a wide array of metabolic syndromes later in life (D.J Barker, 1992; Black et al., 2008; Gluckman eta l., 2008). Along with the fetal genome and other factors, the intra-uterine environment in which the fetus develops is critical to fetal growth and development (Brooks et al. 1995). Maternal nutrition affects placental as well as fetal growth (Belkacemi et al., 2010). IUGR can result from both maternal over nutrition and under nutrition.

Sparse pastures, especially during winters, and drought conditions lead to malnourishment of the cattle. Low quality roughage is not appealing to the microbial flora, hence nutrient supplementation during mid-pregnancy is essential for healthy development of the fetus (J.J. Robinson, 1990). In sheep, flushing (providing the sheep with excess feed) is practiced from 2 weeks prior until the 4^{th} week into breeding without evaluating the body condition score of the ewes in an attempt to increase ovulation (Zimmerman et al., 1960; Flowers et al., 1989). Although flushing increases the number of offspring produced, these offspring often have body weights lower than normal standards (Wootton et al., 1983).

Maternal Under Nutrition and Fetal Development

Poor nutrient availability during gestation results in severe growth retardations in the placenta as well as the fetus (Allen et al., 2002). Cattle that graze un-supplemented during pregnancy suffer from poor placental growth, leading to poor fetal growth and development (Belkacemi et al., 2010). Pregnant women may be undernourished due to lack of adequate availability of nutrients, pregnancies at a very young age or closely spaced pregnancies

(Khoshnood et al. 1998). In pigs, disproportionate nutrition distribution along the uterine horn results in 15 to 20% of the offspring with reduced body weight and reduced performance, which makes them incompetent to survive (Dzuik, 1985). Placental insufficiency, a result of poor placental development does not allow an adequate transfer of nutrients via the placenta to the fetus. Nutrient sensitivity of the fetus is elevated during the peri-implantation period and the period during which there is rapid placental growth. The period of gestation during which the mother is subjected to restricted nutrient availability is critical for the development of the placenta as well as that of the fetus. Suboptimal nutrient availability during early to mid-gestation in sheep increased the placental weight:fetal weight ratio, with increases in placental weight and no increase in the fetal weight (Heasman et al., 1998). Angiogenesis, nutrient transfer capacity and histomorphology are also affected when the nutrient availability is poor (Itoh et al., 2002; Lesage et al., 2002). These factors lead to the birth of offspring that are lighter than normal offspring, often referred to as small for gestational age (SGA). Lack of sufficient nutrient availability during fetal life causes permanent changes in the fetal genome, predisposing the offspring to diseases in its adolescence or adulthood (Waterland et al., 2004). The most evident consequence of restricted maternal diet is the decrease in the size of the offspring observed in rats, pigs, mice, sheep and humans (Lechtig et al., 1975; Dwyer et al., 1994; Ong and Dunger, 2004). Sub-optimal fetal growth and development as a result of poor maternal nutrition also deregulates the secretion of hormones like glucocorticoids, leptin and IGF-1 (Welberg et al., 2000; Toprak et al., 2004). Skeletal muscle is also highly affected by poor maternal nutrition.

Effects of Maternal Malnourishment on Muscle Development

In a study conducted by Zhu et al. in 2006, ewes were subjected to a 50% nutrient restricted diet from d 28 to d 78 of gestation, which in sheep is the early to mid-gestation period. The maternal nutrient restriction did not affect the weight of the longissimus dorsi muscle of the 78d old fetus. However, the total number of muscle fibers in the muscle was significantly reduced (Zhu et al. 2006). Depending on the period of gestation during which there is nutrient restriction, there is a decrease in the primary muscle fibers, secondary muscle fibers or both. Bovine muscle development in the embryo occurs 2 months postconception, so nutrient restriction during the first two months of embryonic life do not affect muscle characteristics (Russel and Oteruelo, 1981). In pigs and guinea pigs, poor maternal nutrition manifested its effects on muscle development by reducing the number of secondary fibers in the offspring (Ward and Stickland, 1991; Dwyer et al., 1994). Restricted nutrient supply within the first fifty days of gestation in ewes decreases the number of primary myofibers formed (Fahey et al., 2004). Restriction from d50 to d140 of gestation reduces the number of secondary muscle fibers (DemİrtaŞ and Özcan, 2012). Zhu et al. (2006) also observed a modification in the fiber-type composition of the longissimus muscles of 78 day-old fetuses when the nutrient availability to the ewe was restricted from d28 of gestation. The study reported an increased expression of type IIb fibers at the expense of type IIa fibers as a result of maternal nutrient restriction. The fiber composition plays a critical role in the function and metabolism of the muscle.

The overall growth and performance of the offspring is affected later in life when muscle development is impaired during gestation. The number of myonuclei in the muscle fibers is also regarded as an indicator of postnatal development of the muscle. Greenwood et al. (1999) noted that sheep fetuses with poor nutrient availability during mid to late gestation had a decreased rate

of cell proliferation, which led them to hypothesize that the number of myonuclei also played a vital role in modulating postnatal growth of muscle fibers in sheep. In rat pups from dams that received 40% reduced nutrition throughout gestation, there was a similar reduction in the number of myonuclei following 3 weeks of rehabilitation with a control diet postnatally (Bayol et al., 2004).

Hypergylcemia and impaired insulin secretion is also reported in lambs from ewes that received 50% of the nutrient requirement from early to mid-gestation (Ford et al., 2007). These lambs exhibited increased body weight accompanied by increased adiposity in the pelvic area at four months of age. The disturbance in the glucose metabolism is attributed to the imbalance in the muscle development and fat regulation in the body (Ford et al., 2007).

In production animals, a primary concern is the deterioration of meat quality with alterations in muscle composition. Marbling is a term used for describing the amount of intramuscular fat content. Appropriate amounts of marbling enhance the juiciness and palatability of meat. The fat content of the muscle is determined by the number and size of the adipocytes (Du et al., 2010a). As the precursor cells for both myoblasts and adipocytes are mesenchymal stem cells, and the period of muscle formation, fibrogenesis and adipogenesis overlap in the embryo, any disruption in the myogenesis signaling may lead to increased adiposity and fibrosity of the muscle, which can negatively affect meat quality (Li et al., 2006). Studies by Rehfeldt and Kuhn in 2006 demonstrated that pigs with poor maternal nutrition during gestation had fewer muscle fibers at birth, which were compensated postantally by increasing the size of the fibers. Larger fibers decreased the quality of meat from these piglets.

Maternal Obesity and Fetal Development

The incidence of obesity has reached alarming levels in the United States and most other developed countries. Rat, sheep and human studies show that both pregnancy and lactation periods are vital to the health of the offspring postnatally (Howie et al., 2008; Bayol et al., 2008; Smith et al., 2009b). Obese rat dams developed oocytes of inferior quality, thus programming the fetus even before fertilization (Minge et al., 2008). Adolescent offspring of obese rat dams demonstrated altered insulin signaling and abnormalities in mitochondrial complex activity (Shelley et al., 2009). These offspring also exhibit hyperinsulinaemia, hyperglycaemia and increased plasma levels of triglycerides, cholesterol and leptin in adulthood (Ramsay et al., 2002; Samuelson et al., 2008; Gupta et al., 2009). In addition, angiogenesis and skeletal muscle development and composition are severely affected due to overfeeding of the mother during gestation (Golzalez-Bulnes et al., 2012).

Effect of Maternal Over Nutrition on Muscle Development

Obesity is often associated with the prevalence of an inflammatory environment, which promotes adipogenesis and inhibits myogenesis. Adipose tissue in obese individuals secretes inflammatory cytokines, which negatively regulate insulin signaling (Hotamisligil et al., 1993). The trigger for the inflammatory response is the excess intake of nutrients by the individual. Elevated expression of Tumor Necrosis Factor α (*TNFα*) mRNA is observed in the adipocytes of obese mice compared with lean controls (Hotamisligil et al., 1995). Subsequent studies have identified increased expression of other inflammatory cytokines, namely interleukins 6 and 1β (IL-6 and IL-1β), and Chemokine (C-C) motif ligand-2 (CCL2; Eder et al., 2009; Arner et al., 2012; Nov et al., 2012). In humans, increased levels of reactive oxygen species (ROS), and

nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) were observed within a few hours of ingestion of a high-fat diet (Aljada et al., 2004). Enhanced phosphorylation of NF-κB was also observed in fetuses from ewes fed an obesogenic diet until midgestation (Tong et al., 2009). The activated form of this protein activates FOXO, which prompts the proliferating stem cells to exit the cell cycle and survive in a quiescent state (Manolagas and Almeida, 2007; Bakkar et al., 2008). The kinases, c-Jun N-terminal kinase (JNK; Hirosumi et al., 2002), inhibitor of κ kinase (IKK; Cai et al., 2005), and protein kinase R (PKR; Balachandran et al., 2000) are overexpressed in obese individuals and their downstream signaling cascades play a major role in initiating the inflammatory pathway. The toll-like receptor 4 (TLR4)/NF-κB pathway involves the phosphorylation of IKK that binds to TLR4 thereby activating NF-κB, which further induces the expression of cytokines like TNFα (Reyna et al., 2008). TLR2 and TLR4, and the NF-κB and JNK pathways were upregulated in late gestation fetuses from ewes that received an obesogenic diet throughout gestation (Yan et al., 2010). Multiple signaling cascades are activated to initiate inflammation in obese individuals. There is also increased infiltration of the adipose tissue with cells of the immune system (Xu et al., 2003), like macrophages (Weisberg et al., 2003), mast cells (Liu et al., 2009), and natural killer T-cells (Ohmura et al., 2010) in individuals with an obesogenic diet, which contributes to the increase in the local and circulating concentrations of cytokines in these individuals. The actual mechanism of this is not yet understood. The inflammatory environment with elevated cytokine levels and infiltrating immune cells is chronic and has severe consequences on the metabolism in many tissues including the adipose tissue and the muscle.

The inflammatory mediators target the insulin signaling pathway resulting in insulin resistance in the adipocytes (Hotamisligil et al., 1994) and a decreased glucose uptake by these

cells (Stephens et al., 1997). This is brought about by the serine phosphorylation of the insulin receptor substrate -1 (IRS-1), which in turn mediates insulin resistance (Morino et al., 2006). In addition to JNK and IKK, which contribute to the inflammation-mediated insulin resistance, the serine/threonine kinase mammalian target of rapamycin (mTOR) has also been reported to negatively phosphorylate IRS-1 (Um et al. 2004). Thus, many signaling pathways mediate the process of making adipocytes insulin resistant. These kinases target the inflammatory cascade and the insulin signaling pathway simultaneously. A negative inhibitor of the cytokine signaling cascade, the suppressor of cytokine signaling (SOCS) family of proteins has been hypothesized to degrade IRS-1 (Rui et al. 2002). The direct links between inflammation and insulin resistance were established with the studies performed on double mutants for the receptor of TNFα that showed improved insulin sensitivity in murine models (Uysal et al., 1998). These studies indicate an alleviation of insulin resistance and improved glucose sensitivity on exposure to a diet with high calorific value (Chiang et al. 2009). Apart from these known effects of the pro-inflammatory mediators on insulin signaling, an imbalance in the expression of the intermediates in the inflammatory pathway can also induce undue lipolysis and has also been observed to inhibit the activation of the peroxisome proliferator-activated receptor ϒ (PPARϒ), a gene that's known to play a vital role in the process of adipogenesis (Medina-Gomez et al., 2007).

Originally it was believed that there were no direct effects of obesity on inflammation in the muscle, but the inflammatory environment prevalent in tissues such as adipose or liver, which are well known targets of the inflammatory pathways, can potentially influence metabolism in the muscle (Cai et al. 2005). A recent study conducted in human subjects proves that there are indeed inflammatory cascades activated in the muscle as a consequence of obesogenic diet (Varma et al. 2009). The adipose tissue is the main origin of systemic

inflammatory response during obesity and produces cytokines like IL-1β, IL-6 and TNF-α (Fried et al., 1998; Kern et al., 2001). However, distinct from low-grade inflammation, the size of the primary myotubes was smaller in offspring born to obese mothers and interfascicular and interfibrillar spaces in these muscles were significantly greater (Tong et al., 2009). These spaces were filled with collagen fibers and adipocytes, which modify the metabolism of the muscle and are detrimental to the taste and quality of the meat (Tong et al., 2009). Huang et al. (2010) studied the effect of maternal obesity throughout gestation on the myocardium and reported increased collagen deposition accompanied by inflammation of the muscular tissue of the heart.

The effect of maternal obesity on fetal muscle development depends on the timing of fetal growth when the offspring is subjected to poor nutrient environment. During mid-gestation, the mesenchymal stem cells are committed to the myoblast, adipocyte or fibrocyte lineage (Zhu et al., 2004). Fetuses from obese mothers favor the commitment of these stem cells to adipogenic or finrogrnic lineages, resulting in a greater amount of adipose tissue and collagen fibers in the muscle (Yan et al., 2011). The increased adiposity of the muscle predisposes the fetus to Type II diabetes postnatally, as the adipocytes gradually induce insulin resistance in the muscle by paracrine signaling (Kim et al., 2000; Aguiari et al., 2008). A controlled postnatal diet may alleviate some of the detrimental effects of poor maternal nutrition by increasing the expression of monocarboxylate transporter proteins and by an improvement in the fatty acid oxidative capacity in the offspring. However, continuation of a high fat diet postnatally, after prenatal exposure of obesogenic diet results in increased body weight, fat mass, plasma insulin and lactate concentrations in the offspring (Simar et al., 2012). A postnatal high fat diet is also associated with reduced satellite cell number and decreased regenerative capacity in mice (Woo et al.,

2011). In livestock excess adipose compromise the healthfulness and palatability of content of the product (Dodson et al., 2010).

Obesityhas also been shown to have indirect effects on the expression of the MRFs, MyoD and myogenin. Embryos from ewes fed with an obesogenic diet (150% NRC requirement) from 60 days prior to mid gestation expressed less β-catenin (Zhu et al., 2008). β-catenin enters the nucleus to upregulate the expression of myoD and myogenin (Miller et al., 1999).

Summary

Impairment of muscle development in the fetus can lead to a poor quality of life in the offspring. There are many factors that act in concert to orchestrate proper muscle development. Many of these factors are interdependent, so disturbing the expression of one factor may cascade into multiple effects. Poor maternal nutrition has an impact on the weight of the offspring, adiposity, myogenesis, and many metabolic pathways. Pre and postnatal myogenesis are affected by poor nutrient availability during the growth of the fetus. The size of the muscles and fibers within an individual muscle, fiber composition of the muscle, adiposity and fibrosity are influence by maternal nutrition. Maternal nutrition also affects the muscle on a molecular level by disturbing the regulation of multiple pathways such as Wnt/β-catenin, Akt, and inflammatory pathways. Maternal nutrition not only affects growth, but also the body composition. In livestock, a main concern is the deterioration in meat quality due to poor nutrient availability during gestation, which translates to higher costs of production and maintenance of these animals by the farmers, and an inferior quality of meat for the consumer.

Objectives

Skeletal muscle is one of the most important tissues affected by maternal nutrition. It is necessary not only for movement and locomotion, but also for regulation of metabolism and glucose homeostasis. Poor nutrient availability during gestation leads to poor development of muscle fibers and an increase in the adiposity and fibrosity of the muscle. These changes in the morphology of the muscle lower the strength and metabolic activities of the muscle. Changes in metabolism during the fetal stage in turn make the fetus susceptible to chronic diseases later in life. In livestock, poor muscle development is detrimental to the quality, palatability and healthfulness of the meat.

Our goal through this research was to explore possible mechanisms by which maternal nutrition affects fetal muscle development. We hypothesized that in lambs from poorly nourished ewes the proteins favoring muscle growth and development would be downregulated and those that inhibit muscle growth and function would be upregulated. Our final objective was to determine the presence of an inflammatory environment in the muscles of lambs from poorly fed ewes. Our hypothesis was that there would be an increase in the expression of pro-inflammatory cytokines as a result of poor maternal nutrition during fetal development.

Our first objective was to study the morphometrics of the semitendinosus muscle to determine the changes in the muscle fiber size and composition. Our hypothesis was that poor maternal nutrition would lead to a modification in the size of the muscle fibers as well as composition of muscles. The second objective of our research was to analyze the expression of the proteins that play vital roles in regulating muscle growth, development and the various metabolic functions of the muscle.

Materials and Methods

Animals

For the study, 36 multiparous Dorset (n=25), Shropshire (n=7), and Southdown ewes (n=4) were selected from the University of Connecticut flock, following the confirmation of twin pregnancy by ultrasound. Ewes were individually housed in pens and assigned to one of the following diets: Control (CON); 100% of National Research Council (NRC, 1985) requirements, Overfed (OVER); 140% of NRC requirements or Restricted (RES); 60% of NRC requirements. The diet, consisting of pelleted feed and enrichment with straw once a day, was determined on the body weight basis and was reviewed weekly. Ewes were maintained on this diet until parturition. The lambs born were allowed to nurse to obtain colostrum. If sufficient colostrum was unavailable, lambs were administered a commercially available supplement for colostrum (Lamb's Choice Total Colostrum; Saskatoon Colostrum Co; Saskatoon, Canada). One lamb per ewe was retained for study and the rest were returned to the University of Connecticut flock with the ewes. The lamb chosen for study was the larger of the two if both offspring were of the same gender. The male offspring was chosen in the event of a gender difference. There were eighteen lambs chosen for the study fro each time point. One group of lambs (n=6 per diet group) was euthanized within 24 hours of birth. The remaining 18 lambs were maintained on a control milk replacer diet (Land O' Lakes Animal Milk Product Company; Shoreview, MN) via bottle-feeding. The lambs were weighed twice a week and the milk replacer amount (calculated to 7% of body weight) was modified accordingly. Along with the milk replacer lambs also had *ad libitum* access to creep feed (Lamb BT, Blue Seal Feeds; Litchfeild, CT) and second cutting hay. Lambs were weaned from the bottle at 2 months of age and were necropsied at 3 months of age. Lambs were euthanized with intravenous injection of Beuthanasia-D solution.

Muscle Sample Collection

Following euthanasia, a portion of the semitendinosus muscle was removed, embedded in optimal cutting temperature compound (O.C.T., Tissue-Tek, Sakura Finetek USA Inc., CA), and frozen for muscle morphometric analysis. Small portions of the muscle were snap-frozen in liquid nitrogen. The samples were stored at -80°C until analysis.

Immunohistochemistry

The muscle portion that was embedded in O.C.T. was sectioned on a Leica CM 3050S cryostat maintained at -20°C. The sections were transferred onto a pre-cleaned glass slide. Slides were stored at -80°C till further use and thawed at room temperature (RT) then warmed on a heating block (45 °C) for 1-2 min to keep the sections adherent. Following this, sections were outlined with a narrow lining of petroleum jelly to retain the solutions on the section. The sections were then fixed with 4% paraformaldehyde solution for 20 min at RT, followed by three 5-minute washes with phosphate-buffered saline (PBS). The sections were then incubated for 20 min in blocking solution (5% Horse serum in PBS), followed by three 5-minute washes with PBS. These sections were stained with Wheat Germ Agglutinin (1:50, WGA - Alexa Fluor 594, Invitrogen, CA, USA) for 120 min in dark at 4°C to stain the sarcolemma of muscle fibers. Alternatively, for staining Type I, Type IIa, Type IIb fibers, and the sarcolemma of the muscle fibers, the sections were stained with the BAD-5 (1:10, Type I), SC-71 (1:10, Type IIa), BFF3 (1:10, Type IIb; Developmental Studies Hybridoma Bank, University of Iowa) and anti-dystrophin (1:50, Thermo Scientific, MA, USA) overnight at 4°C in humidified boxes. To remove unbound primary antibody the slides were washed 3X with PBS, followed by incubation with the respective fluorescently tagged secondary antibody (1:250, Alexa Fluor 488, 594 and

633, Invitrogen, CA, USA) for 60 min in the dark. The slides were then washed 3X with PBS, cover-slipped with glycerol (9:1 glycerol:PBS), and microscopically observed (Zeiss Axiovert Widefield Microscope). The images were obtained using a Hamamatsu Orca ER camera (model number: C4742-95-12ERG) and were saved and modified using the Open Lab software. Further analysis of these images was performed with the software Image-J (National Institutes of Health).

Western Blotting

Snap-frozen muscle was transferred to a 2 mL centrifuge tube containing 250 μL of Western Buffer (62.5 mM Tris HCl, 25 % glycerol, 10% SDS w/v) for homogenization. The tubes were balanced in the blocks of the TissueLyser II homogenizer (Qiagen Sample and Assays Technology, USA) and homogenized for 5 minutes at 30 cycles/sec. Following the first homogenizing, the bead was removed from the tube with sterile forceps, and the tube was centrifuged at 4^{o}C for 5 min at 15,000 rpm. The supernatant obtained was pipetted into a sterile tube, labeled and stored at -20^{o}C until further use. To the 2 mL centrifuge tube with the partially homogenized muscle, another sterile bead and another 250 μL of Western Buffer was added, and homogenization was repeated as described above. Protein concentration was determined by spectrophotometry using a Thermo Scientific Nanodrop 1000 (Thermo Scientific, MA, USA).

Samples (27.18 μg) were denatured at 75^{o}C for 5 min prior to loading on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis along with a protein standard. Gels were run at 150 V and 15 Amps until the tracking dye front crossed the stacking gel. The voltage and the amps were then increased to 200 V and 30 Amps until the dye front reached the bottom of the resolving gel. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon Transfer Membrane, Millipore Corporation, MA, USA) at 100 V for 90 min. The

membrane was stained with Ponceau S to confirm transfer. The blot was then incubated in blocking buffer (Licor Biosciences, NE, USA) for 60 min with constant shaking at RT. The membrane was then incubated in the respective primary antibodies (***Table 1***) in 10 mls of blocking buffer with 0.1% Tween 20 overnight with constant shaking at 4^{o}C. The next day the blot was subjected to 3X 5-minute washes with PBS and Tween 20 (PBS-T), followed by incubation with the respective secondary antibody (***Table 1)*** for 60 min in the dark. The blot was then washed four times with PBS-T, scanned with the Licor Odyssey Scanning system, and analyzed with Image Studio (Licor Biosciences, NE, USA).

RNA Isolation and PCR

RNA was isolated by the standard phenol/chloroform method (Liu and Harada,2013). One microgram of RNA was reverse transcribed to cDNA in a 20 μL reaction (Superscript III, Invitrogen). Two microliters of cDNA was amplified with gene specific primers (***Table 2***). PCR products were visualized following electrophoresis through 2% agarose gels containing ethidium bromide to confirm product size. Real time PCR was performed using the same primers. Thermal cycling parameters included denaturation for 10 min at 95°C, followed by 60 cycles of 15 sec at 95°C and 1 min at 60°C. Serial dilutions of pooled samples were used to generate standard curves to ensure generation of cycle threshold values that were within the linear range of amplification (Castellani et al., 2004). 18S gene was used as a housekeeping gene and the expression of the inflammatory genes is represented relative to 18S expression.

Statistical Analysis

The PROC MIXED procedure of SAS was used to analyze the data as a randomized

complete block design of experiment and lsmeans was used to compare means of individual treatment effects. *P* value of less than 0.05 was considered significant.

Table 1. Antibody specifications

Antibody	Dilution	Source
Akt	1: 200	Cell Signaling Technology
Phospho-Akt (Thr 308)	1: 1000	Cell Signaling Technology
Phospho-Akt (Ser 473)	1: 1000	Cell Signaling Technology
Myostatin (GDF-8 (N-19)-R: sc-6885-R	1: 200	Santa Cruz Biotechnology
Follistatin (H-114): sc-30194	1: 200	Santa Cruz Biotechnology
Tubulin-α Ab-2 (Clone DM1A)	0.5 µg/ml	Thermo Scientific
Goat Anti-mouse IRDye 800 CW	1:2000	Licor Biosciences
Goat Anti-rabbit IRDye 680 RD	1:2000	Licor Biosciences

Table 2. Primer homology

Gene	Primer	Product length (bp)	Primer region homology, %[1]	Homology Bovine vs. Ovine, %[1]
IL-6	Bt03211905_m1	115	96	96
TNFα	Bt03259154_m1	84	95	94
IL-1β	Bt03212741_m1	71	95	95

[1]Bovine vs. ovine

Results

Maternal nutrition affects the cross sectional area of muscle fibers

At birth, the cross sectional area (CSA) of muscle fibers from the semitendinosus muscle of the OVER and RES lambs was 47% and 57% greater than lambs from CON ewes, respectively (Figure 1; CON: 553.3± 62.8 μm^2; OVER: 817.1 ± 26.0 μm^2; RES: 871.5 ± 114.4 μm^2; $P_{CON\ vs.\ OVER} = 0.025$, $P_{CON\ vs.\ RES} = 0.009$). At three months of age the CSA of the lambs from OVER ewes was 17% smaller and that of lambs from RES ewes was 15% smaller than the CSA of lambs from CON ewes (Figure 2; CON: 2139.5 ± 40.3 μm^2; OVER: 1775.6 ± 65.1 μm^2; 1809.4 ± 90.1 μm^2; $P < 0.0001$.)

Maternal nutrition alters the fiber type composition of the semitendinosus muscle

At birth, OVER and RES lambs had 6.8% and 7.8% more Type IIb fibers respectively as compared with the CON lambs, with a similar reduction in the number of Type I fibers compared with the CON lambs (Figure 3, Table 3; $P_{CON\ vs.\ OVER}$ and $P_{CON\ vs.\ RES} < 0.0001$). At 3 months of age, there was no difference in the composition of the fibers in the muscles of OVER or the RES lambs as compared with the CON lambs (Figure 4, Table 3; $P_{CON\ vs.\ OVER} = 0.2$, $P_{CON\ vs.\ RES} = 0.06$). There were no Type IIa fibers identified in the semitendinosus muscle of any of the lambs. There was also no significant difference in the effect of maternal nutrition on the CSA of Type I or Type IIb fibers in lambs necropsied at birth or at three months of age (Figures 5; $P_{CON\ vs.\ OVER} < 0.04$, $P_{CON\ vs.\ RES} < 0.02$; and Figure 6; $P_{CON\ vs.\ OVER} = 0.2$, $P_{CON\ vs.\ RES} = 0.06$, Table 4).

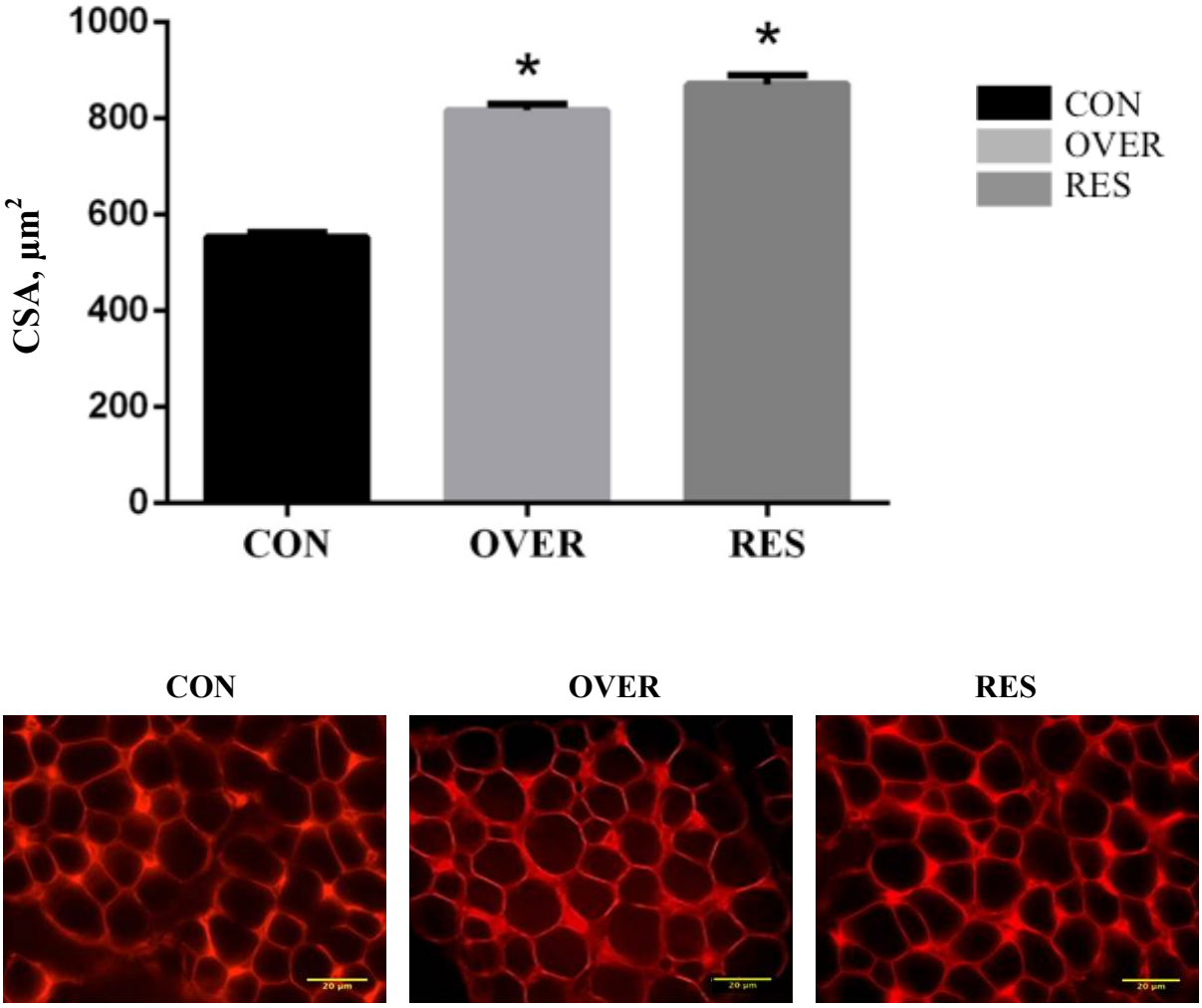


Figure 1. **Poor maternal nutrition increases the CSA of muscle fibers in the semitendinosus muscle of lambs at birth**. Semitendinosus muscle was stained with Wheat Germ Agglutinin (WGA) to delineate fiber membranes and the CSA was measured. The data are presented as mean ± SEM. CON – lambs from control-fed ewes (n=3); OVER – lambs from overfed ewes (n=3); RES - lambs from restricted-fed ewes (n=3); and CSA- cross sectional area. * indicates a significant difference at $P < 0.05$ vs. CON

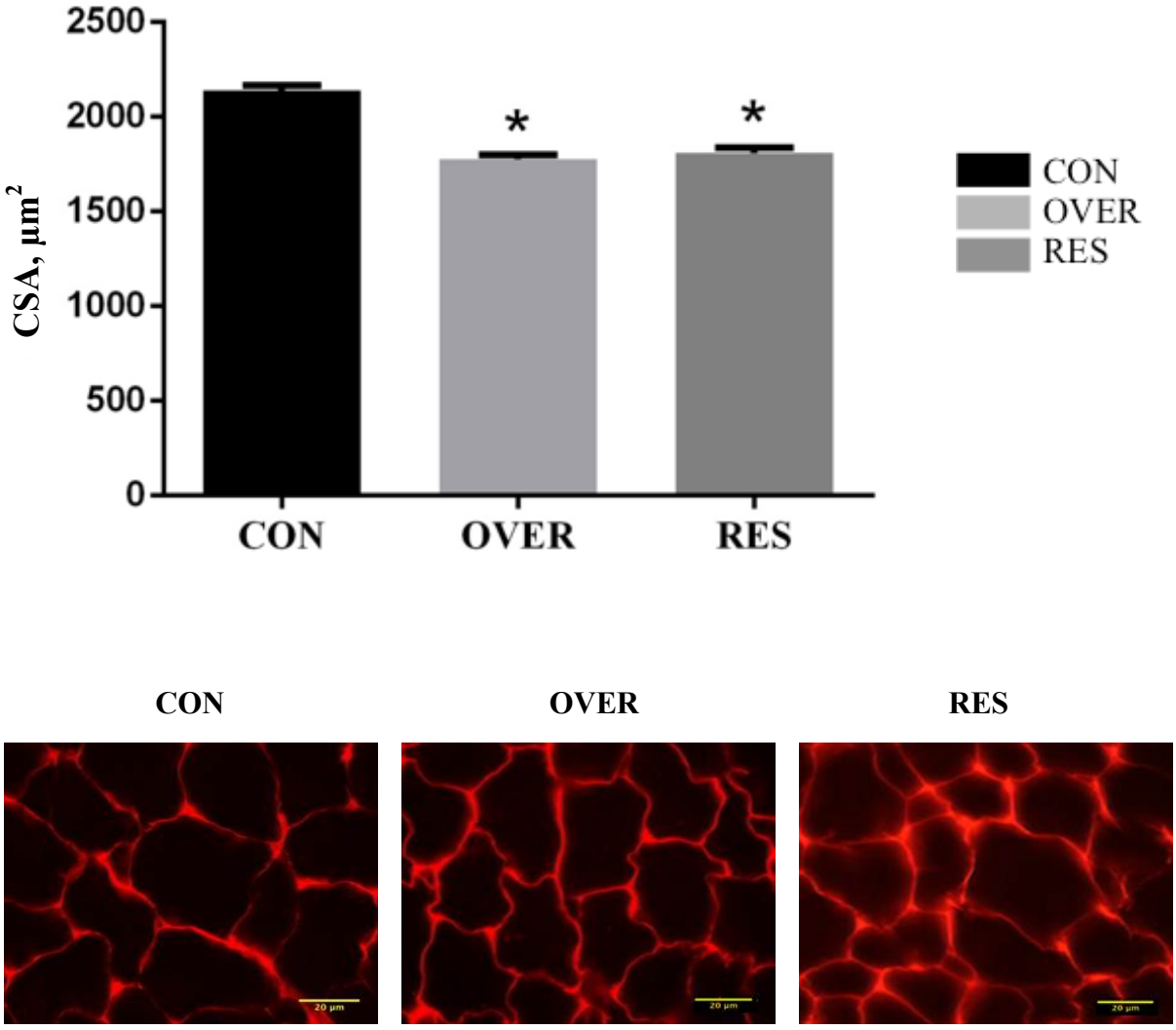


Figure 2. **Poor maternal nutrition impairs growth of muscle fibers in the semitendinosus muscle of lambs necropsied at 3 months of age**. Semitendinosus muscle was stained with Wheat Germ Agglutinin (WGA) to delineate fiber membranes and CSA was measured. The data are presented as mean ± SEM. CON – lambs from control-fed ewes (n=3); OVER – lambs from overfed ewes (n=3); RES - lambs from restricted-fed ewes (n=3); and CSA- cross sectional area. * indicates a significant difference at $P < 0.0001$ vs. CON.

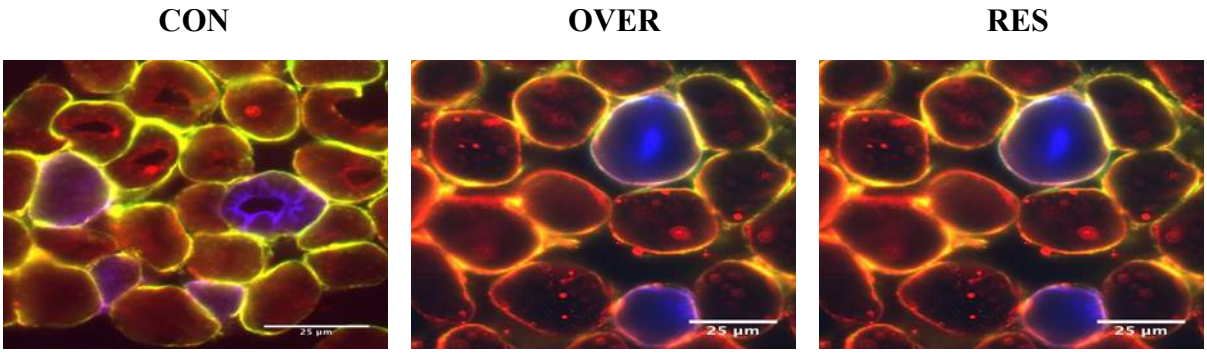


Figure 3: **The composition of muscle fibers in the semitendinosus muscle of lambs is changed at birth.** Semitendinosus muscle was stained for Type I (blue), Type IIa (green), and Type IIb (red) fibers, and dystrophin (yellow). There were no Type IIa fibers detected in any of the samples. CON – lambs from control-fed ewes (n=3); OVER – lambs from overfed ewes (n=3); RES - lambs from restricted-fed ewes (n=3).

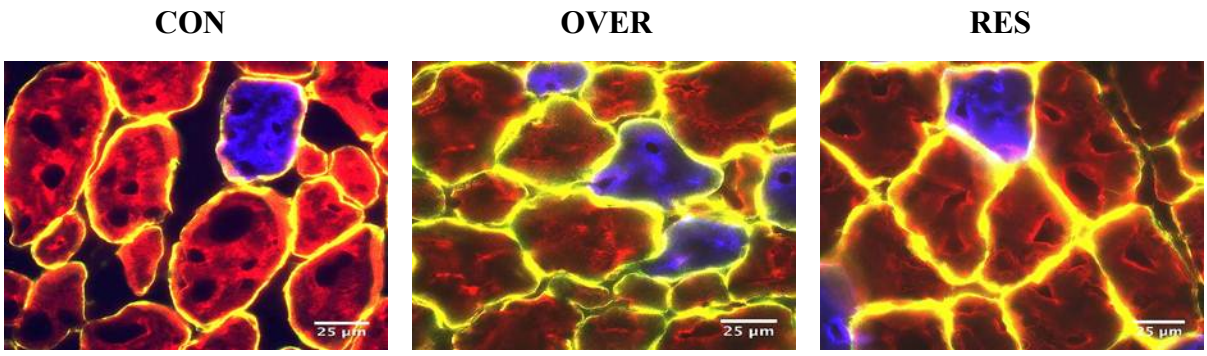


Figure 4: **The composition of muscle fibers in the semitendinosus muscle of lambs at 3 months of age is not affected by poor maternal nutrition.** Semitendinosus muscle was stained for Type I (blue), Type IIa (green), and Type IIb (red) fibers, and dystrophin (yellow). There were no Type IIa fibers detected in any of the samples. CON – lambs from control-fed ewes (n=3); OVER – lambs from overfed ewes (n=3); RES - lambs from restricted-fed ewes (n=3).

Table 3. **Fiber composition of the semitendinosus muscle**

	Birth			3 Months		
	Type I, %	Type IIa, %	Type IIb, %	Type I, %	Type IIa, %	Type IIb, %
CON	19.5 ± 1.0	N.D.	80.5 ± 1.0	16.2 ± 2.8	N.D.	83.8 ± 2.8
OVER	12.7 ± 0.4	N.D.	87.3 ± 0.4	19.0 ± 1.8	N.D.	81.0 ± 1.8
RES	11.7 ± 1.4	N.D.	88.3 ± 1.4	20.5 ± 1.6	N.D.	79.5 ± 1.6

N.D. – Not Detected

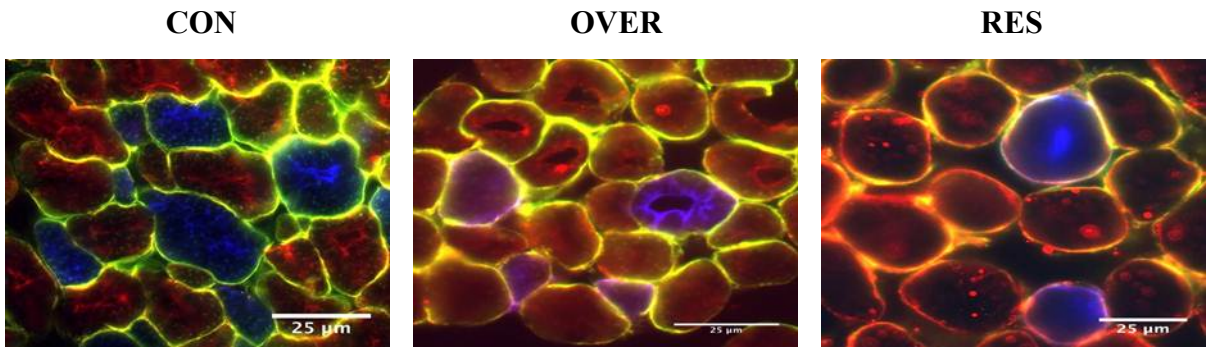


***Figure 5*: Poor maternal nutrition does not affect the CSA of one specific fiber type at birth.** Semitendinosus muscle was stained for Type I (blue), Type IIa (green), and Type IIb (red) fibers, and dystrophin (yellow) and cross sectional area of fibers was measured. There were no Type IIa fibers detected in any of the samples. CON – lambs from control-fed ewes (n=3); OVER – lambs from overfed ewes (n=3); RES - lambs from restricted-fed ewes (n=3).

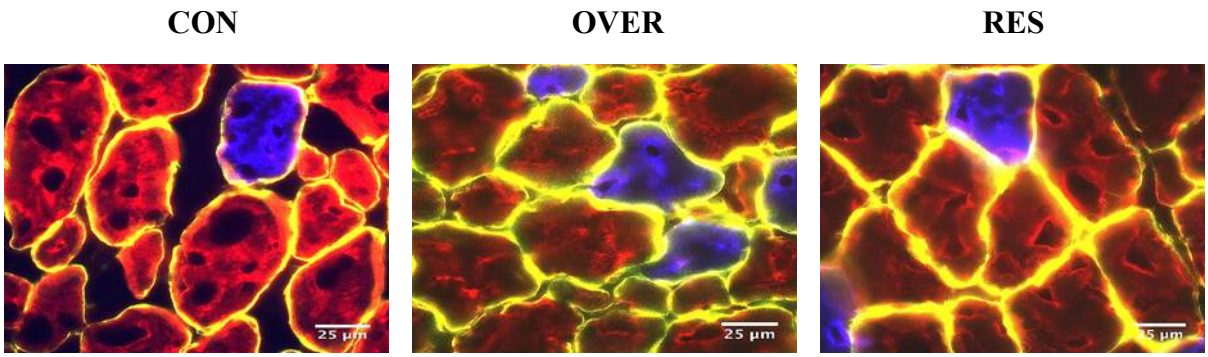


***Figure 6*: Postnatal growth of all fibers is affected similarly by poor maternal nutrition**. Semitendinosus muscle was stained for Type I (blue), Type IIa (green), and Type IIb (red) fibers, and dystrophin (yellow) and cross sectional area of fibers was measured. There were no Type IIa fibers detected in any of the samples. CON – lambs from control-fed ewes (n=3); OVER – lambs from overfed ewes (n=3); RES - lambs from restricted-fed ewes (n=3).

***Table 4.* Fiber-specific CSA of muscle fibers in the semitendinosus muscle of lambs**

	Birth			3 Months		
	Type I, μm^2	Type IIa, μm^2	Type IIb, μm^2	Type I, μm^2	Type IIa, μm^2	Type IIb, μm^2
CON	307 ± 57.7	N.D.	337.3 ± 24.5	1791.1 ± 240.9	N.D.	1934 ± 69.0
OVER	570.7 ± 46.5	N.D.	570.2 ± 43.3	1099.6 ± 126.5	N.D.	1109.7 ± 11.8
RES	515.2 ± 32.2	N.D.	622.0 ± 8.5	1028.2 ± 27.0	N.D.	1151.3 ± 21.6

N.D. – Not Detected

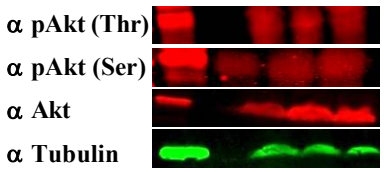
Maternal nutrition affects the phosphorylation of Akt at the serine residue

There was no significant difference in the protein expression of Akt at birth (Figure 7G; CON: 1.0 ± 0.2; OVER: 0.7 ± 0.1; RES: 0.5 ± 0.2) or at 3 months of age (Figure 7H; CON: 1.0 ± 0.3; OVER: 1.1 ± 0.3; RES: 0.8 ± 0.1; $P = 0.7$) or of phosphorylated Akt at threonine 308 [pAkt (thr)] at birth (Figure 7C; CON: 1.0 ± 0.8; OVER: 1.4 ± 0.5; RES: 2.4 ± 0.4; $P_{CON\ vs.\ OVER} = 0.6$, $P_{CON\ vs.\ RES} = 0.1$) or three months of age (Figure 7D; CON: 1 ± 0.5; OVER: 0.5 ± 0.3; RES: 1.0 ± 0.3; $P_{CON\ vs.\ OVER} = 0.5$, $P_{CON\ vs.\ RES} = 0.9$*)* as a consequence of poor maternal nutrition during gestation. There was also no significant effect of poor maternal nutrition on the expression of pAkt, phosphorylated at serine 473 at birth (Figure 7E; CON: 1.0 ± 0.3; OVER: 1.5 ± 0.6; RES: 1.9 ± 0.9; $P_{CON\ vs.\ OVER} = 0.7$, $P_{CON\ vs.\ RES} = 0.5$). However there was a 4.5 fold increase observed in the expression of pAkt(Ser) in RES lambs as compared with CON lambs at three months of age (Figure 7F; RES: 4.5 ± 1.2 ,CON: 1.0 ± 0.3; $P_{CON\ vs.\ RES} = 0.006$). OVER lambs at 3 months of age showed no significant difference in the expression of pAkt(Ser) compared with CON lambs (OVER: 1.3 ± 0.5; $P_{CON\ vs.\ OVER} = 0.8$).

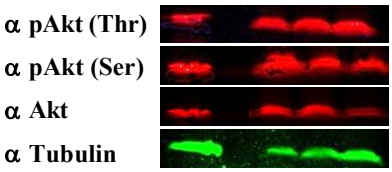
Maternal diet does not affect myostatin or follistatin protein expression

There was no effect of poor maternal nutrition on the expression of the precursor or active myostatin monomer at birth (Figure 8A, C and E; $P \geq 0.5$). The active dimeric form was not expressed at birth. There was no effect of ewe diet on the expression of the myostatin precursor, active dimer or active monomer at 3 months (Figure 8B, D, F and G; $P \geq 0.5$).There was no effect of poor maternal nutrition on the expression of the follistatin protein at birth (Figure 9A and C; $P \geq 0.2$) or 3 months (Figure 9B and D; $P \geq 0.6$).

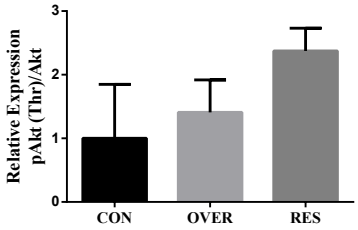
A.


+
C
O
R
α pAkt (Thr)
α pAkt (Ser)
α Akt
α Tubulin

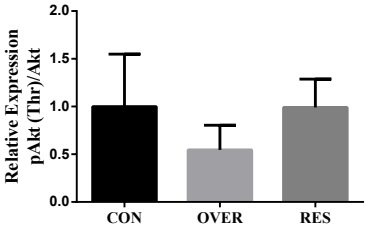
B.


+
C
O
R
α pAkt (Thr)
α pAkt (Ser)
α Akt
α Tubulin

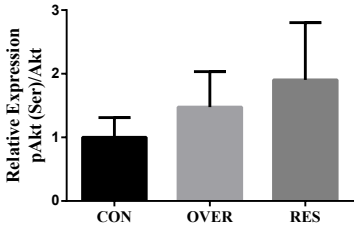
C.


Relative Expression
pAkt (Thr)/Akt
3
2
1
0
CON
OVER
RES

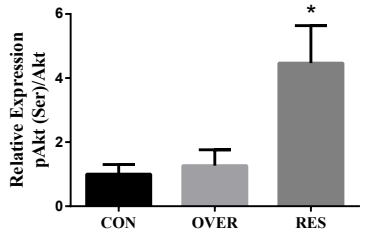
D.


Relative Expression
pAkt (Thr)/Akt
2.0
1.5
1.0
0.5
0.0
CON
OVER
RES

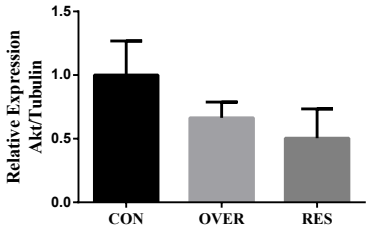
E.


Relative Expression
pAkt (Ser)/Akt
3
2
1
0
CON
OVER
RES

F.


Relative Expression
pAkt (Ser)/Akt
6
4
2
0
*
CON
OVER
RES

F.


Relative Expression
Akt/Tubulin
1.5
1.0
0.5
0.0
CON
OVER
RES

H.

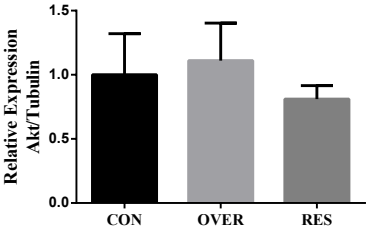

Relative Expression
Akt/Tubulin
1.5
1.0
0.5
0.0
CON
OVER
RES

Figure 7: **Poor maternal nutrition increases the expression of pAkt (Ser)** Representative western blots for phosphorylated Akt (Thr 308 and Ser 473), total Akt, and tubulin (loading control) in the muscle of lambs necropsied at birth (A) and 3 months of age (B). There was no difference in the expression of the pAkt (Thr) protein (C and D) in relation to total Akt (G and H) at birth or 3 months. There was also no difference in the expression of pAkt (Ser) protein (E) in relation to total Akt at birth. At 3 months there was a significant increase in the expression of pAkt (Ser) in RES lambs compared with CON lambs (F). Data are represented as mean ± SEM.* $P = 0.006$. C/CON – lambs from control-fed ewes (Birth: n = 4, 3 months: n = 3); O/OVER – lambs from overfed ewes (Birth: n = 5, 3 months: n = 3); R/RES - lambs from restricted-fed ewes (Birth: n = 5, 3 months: n = 4); and + - Positive control.

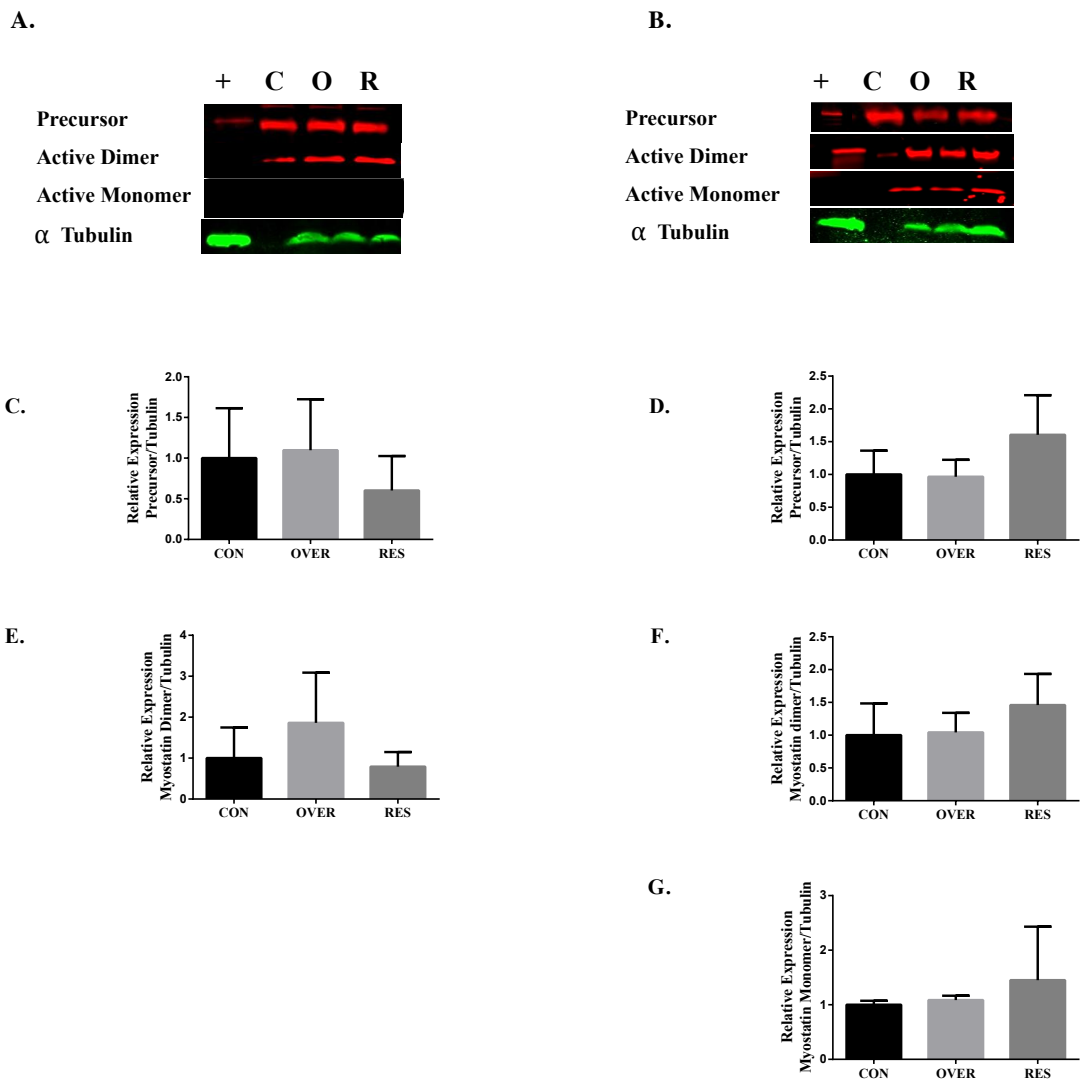


Figure 8: **Poor maternal nutrition does not affect the expression of myostatin protein at birth or 3 months of age.** Representative western blots for the 52 kDa precursor, the 26 kDa dimer and the 13 kDa monomeric isoforms of myostatin at birth (A) and 3 months of age (B). Expression of the precursor (C and D), active dimer (F), active monomer (E and G) forms of myostatin at birth and 3 months of age respectively. The data are represented as mean ± SEM. C/CON – lambs from control-fed ewes (Birth: n = 4, 3 months: n = 3); O/OVER – lambs from overfed ewes (Birth: n = 5, 3 months: n = 3); R/RES - lambs from restricted-fed ewes (Birth: n = 5, 3 months: n = 4); and + - Positive control.

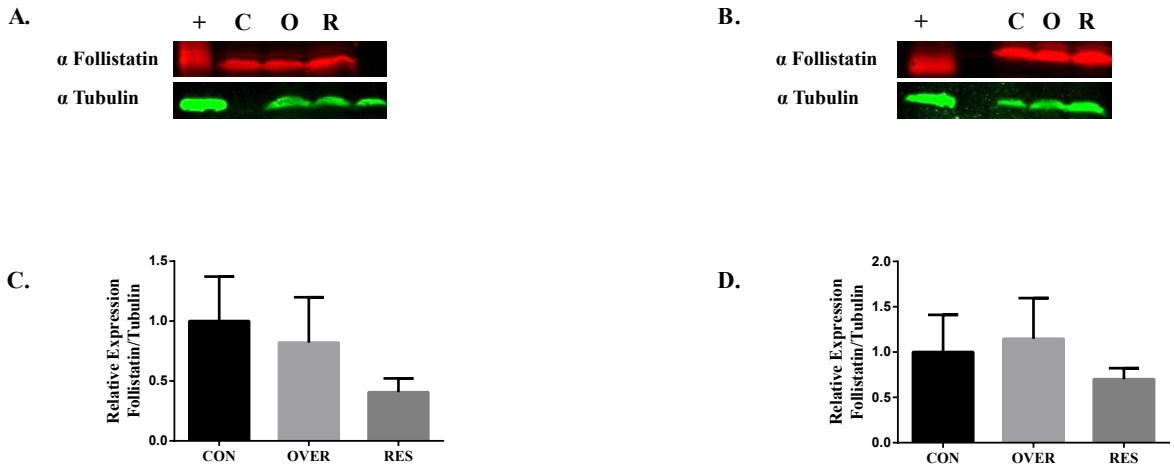


Figure 9: **Follistatin protein expression is not affected by poor maternal nutrition.** Representative western blots for the 35 kDa follistatin protein from the semitendinosus muscle of lambs at birth (A) and 3 months (B). Expression of the protein at birth (C) and 3 months of age (D). C/CON – lambs from control-fed ewes (Birth: n = 4, 3 months: n = 3); O/OVER – lambs from overfed ewes (Birth: n = 5, 3 months: n = 3); R/RES - lambs from restricted-fed ewes (Birth: n = 5, 3 months: n = 4); and + - Positive control.

Poor maternal nutrition during gestation does not increase inflammatory cytokines in the muscle of the offspring

There was no significant effect of poor maternal nutrition on the expression of TNFα at birth (Figure 10; CON: 1.0 ± 0.3; OVER: 0.7 ± 0.2; RES: 0.5 ± 0.2; $P = 0.4$) or three months (CON: 1.0 ± 0.1; OVER: 2.0 ± 0.7; RES: 1.5 ± 0.6; $P = 0.3$), or IL-6 at birth (CON: 1.0 ± 0.1; OVER: 0.9 ± 0.1; RES: 1.1 ± 0.1; $P = 0.5$) or three months - CON: 1.0 ± 0.1; OVER: 1.3 ± 0.1; RES: 2.1 ± 0.6; $P = 0.3$). IL-1β could not be detected.

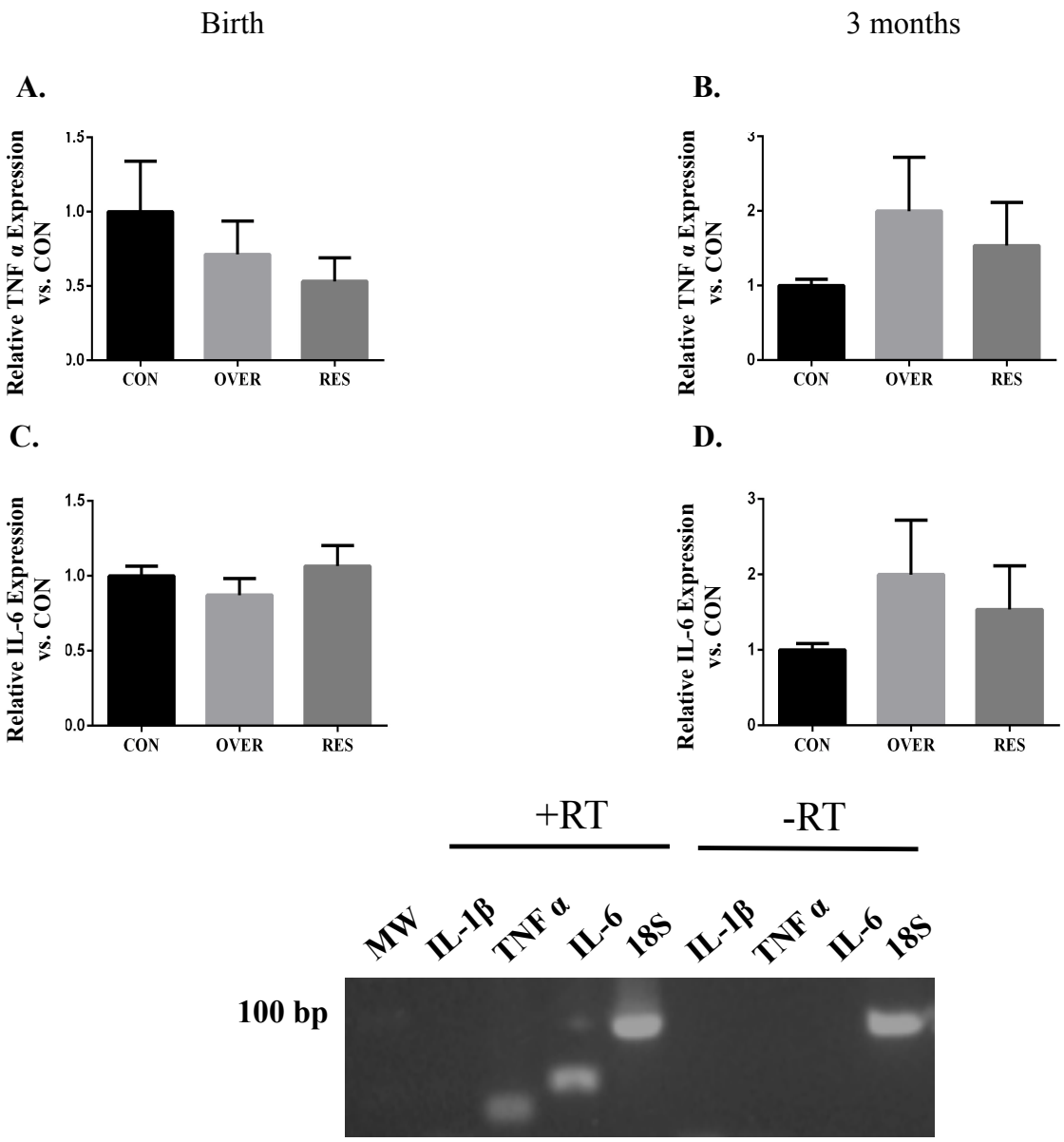


Figure 10: **Poor maternal nutrition does not increase inflammatory genes in the muscle.** Total RNA from lamb semitendinosus muscle was isolated and subjected to real time PCR analysis for TNFα, IL-6 and IL-1β. There was no effect of maternal diet on TNFα (A and B) and IL-6 (C and D) at birth or 3 months of age. No expression of IL-1β was observed via real time or end-point PCR (E). The data is represented as mean ± SEM. C/CON – lambs from control-fed ewes (Birth: n = 5, 3 months: n = 5); O/OVER – lambs form overfed ewes (Birth: n = 4, 3 months: n = 3); R/RES - lambs from restricted-fed ewes (Birth: n = 5, 3 months: n = 4).

Discussion

Most of the research performed to date on the effects of poor maternal nutrition during gestation has focused on the ill effects of under nourishment or over nourishment exclusively (Bayol et al., 2004; Daniel et al., 2007; Ford et al., 2007; Shelley et al., 2009). The research described here is novel in that our experimental design encompassed both these poor nutrient statuses studied at two major periods of life, the neonatal stage, which is influenced by the processes and the metabolism of the offspring during gestation, and the early postnatal stage, which provides a clear picture of the metabolic and physiological adaptations of the offspring from a fetal life to an independent life. The sheep was chosen as our model of study due to its physiological similarity to humans including placental development (Barry and Anthony, 2009) and muscle development pre and postnatally (Maltin et al., 2008).

Poor maternal nutrition during gestation retards the growth of muscle fibers postnatally

The total number of muscle fibers in mammals is predetermined at birth, hence the gestational development of muscle fibers is crucial for normal muscle functions and metabolism in an adult animal (Jue et al., 1989; Greenwood et al., 2000; Meyer et al., 2002; Nissen et al., 2003). When the fetus is subjected to a lower nutritional plane than required for normal growth and metabolism, the body partitions the available nutrients to the more vital organs first, like the heart and the brain, before the skeletal muscle is nourished (Sparks et al., 1983). The fetuses developing in a rodent dam fed to the point of obesity had retarded myogenesis, evidenced by the decreased expression of myogenic regulatory factors like MyoD, mainly as the mild inflammatory environment in the dam upregulates adipogenesis (Bakkar et al., 2008; Bayol et al., 2008). The changes that occur in the fetus during gestation cause both reversible and

irreversible changes in the metabolism predisposing it to conditions like obesity, insulin resistance and muscle weakness with age.

The number and size of the muscle fibers decrease in lambs from poorly nourished ewes, especially when the nutrient availability is not optimal from early to mid-gestation, the phase when secondary myofibers are formed (Zhu et al., 2004). We studied the semitendinosus muscle primarily because the fibers of the muscle are uniformly oriented and the chemical properties of the muscle are relatively uniform throughout the tissue (Basaran-Akgul et al., 2008). In our study, we observed that at birth, lambs from poorly nourished ewes (both overfed and underfed) had larger muscle fiber CSA compared with those of lambs from ewes fed with a CON diet. This observation in the CSA difference reiterates the findings of a study conducted in the late 1980's. Cerisuelo et al. (1989) studied the effect of increased sow nutrition midgestation on the meat quality in piglets. They observed larger fibers in piglets from sows that ate an obesogenic diet. This adaptation is likely a preparation for a probable poor nutrient environment in postnatal life, which is known as fetal programming. The decrease in the number of fibers due to suboptimal nutrient availability is compensated for by an increase in size of the muscle fibers possibly in an attempt to perform all functions of the muscle as efficiently as possible. At the age of three months there was a remarkable change in the CSA trend, in that lambs from poorly fed ewes had fibers with smaller CSA compared with those of lambs from CON ewes. This was unexpected as previous studies observed no effect of maternal nutrition on the postnatal development of the muscle in piglets over 4 weeks of age (Nissen et al., 2003) and others showed an increase in muscle fiber CSA postnatally (Zhu et al, 2004) in the fetuses exposed to poor nutritional environment during gestation as compared with lambs from CON ewes. Our findings indicate that the growth of muscle fibers is retarded when the fetus suffers poor nutrient conditions during

the development of muscle fibers. Inadequate transfer of macro and micronutrients through the placenta during gestation could lead to long term impairment in the major pathways like IGF-1, which plays a vital role in glucose metabolism and mediates its effect via secondary signaling molecules like Akt (McDonald et al., 2007). Other mechanisms may include the methylation of genes encoding vital proteins like the myogenic regulatory factors, which would then result in suboptimal muscle fiber growth and development postnatally (Laker et al., 2013). Hence the muscle fibers that were significantly larger in lambs from poorly nourished ewes could not develop as large and efficiently as the fibers from lambs from CON ewes.

The poor postnatal development of muscle fibers, as evidenced by the CSA analysis of muscle fibers encouraged us to analyze the changes in the CSA of individual fibers on the basis of their fiber type to examine if maternal nutrition affected a specific fiber type more severely than the rest. The lack of difference in the CSA of either Type I or Type IIb fibers at birth or three months of age suggests that maternal nutrition affects all the types of fibers in a similar way. Although we stained muscle sections for the Type IIa fibers, we did not observe any expression of Type IIa fibers in any of our animals. We suggest that the early postnatal stage of three months was not time enough for the complete development of all the types of muscle fibers. The body produces only those fiber types that are necessary and sufficient for the structural and metabolic functions of the muscle. However, there are some studies on 3 month (Daniel et al., 2007) and 8-month (Zhu et al., 2006) old lambs that note the expression of Type IIa fibers early in life. However, Daniel et al. studied the muscle characteristics in the semitendinosus muscle of piglets when they weighed 104 kg. The development of muscle fibers may vary to an extent between species. The study by Zhu et al., (2006) studied the longissimus

dorsi muscle of 8 month old lambs. Age as well as the muscle could possibly contribute to the development of various fibers types catering to the needs of the body.

Maternal nutrition affects the composition of muscle fibers in the semitendinosus muscle

The analysis of the composition of fiber types in the semitendinosus muscle at birth showed a significant increase in the percent of Type IIb in lambs from poorly nourished ewes at the expense of Type I fibers at birth. At three months of age, there was no significant difference in the composition of muscle fibers in the muscle of lambs from poorly nourished ewes as compared with those from CON ewes. This suggests that the animals subjected to a poor nutrient environment during crucial periods of fetal growth are programed to achieve the best efficiency from fewer myofibers. This also conforms to Darwin's 'survival of the fittest' theory, as the semitendinosus muscle is involved in the swift movements of the animal, like fleeing at the sight of a predator, or competing with other sheep in the herd for food. In order that the lamb is competent, the percent of Type IIb muscle fibers in the muscle is increased. The body anticipates poor nutrient availability postnatally as well and braces itself to survive by increasing the number of Type IIb fibers. Postnatally the availability of an optimal diet likely allowed for some Type IIb fibers to switch to Type I fibers. As the animal grows, there is also enhanced accumulation of adipose tissue in the muscle and in the viscera, which is a major source of energy for Type I fibers (Jarvis et al., 1996). Our observation differs from that of Fahey et al. (2005b) who observed no difference in the number of Type IIb fibers at the neonatal age, and Zhu et al. (2006) whose data indicated an increase in Type IIb in 8-month old offspring of lambs from ewes subjected to a RES nutrient supply. The study by Fahey et al. in 2005 restricted the diet of the ewes only during d30 to d70 of gestation, following which the diet was returned to 100% of NRC requirements. This nutrient restriction affected the formation of muscle fibers in

the offspring, as d30 to mid gestation is the phase of muscle development from the dermomyotome, which marks the beginning of primary myogenesis (Wilson et al., 1992). Availability of adequate nutrient supply during the latter half of gestation could have induced a switch from Type IIb to IIa or I. The sudden increase from 50% to 100% of NRC requirements could have signaled the body of excess nutrient availability. Also with progression from the fetal stage, there is more adipose tissue development, which is the fuel source for Type I fibers (Briand et al., 1981). The findings of Zhu et al. (2006) under similar maternal dietary restriction condition and period as Fahey et al.'s research reported enhanced expression of Type IIb fibers in lambs from poorly nourished ewes at a much later postnatal age of 8 months. This could imply serious dysregulation of the expression of the types of myosin heavy chain as the body ages, despite availability of control diet after muscle formation.

Postnatal phosphorylation of Akt in the muscle is affected by poor maternal nutrition

Akt is a protein kinase that plays a pivotal role in many cell processes including proliferation, glucose metabolism and angiogenesis (Sabatini et al., 1994; Fayard et al., 2005). Our study compared Akt protein expression in the three groups of animals in relation to its role in cell growth and muscle mass. To the best of our knowledge, this is the first study that has analyzed the effects of maternal nutrition on Akt in the muscle. There was no significant change in the expression of Akt protein in CON lambs. However, there was a lot of variation in the data that may be improved by increasing the number of animals in each group. The trend in the data suggests that there is decreased Akt in lambs from poorly nourished ewes necropsied at birth, which may or may not be rectified when nourished with a healthy feed postnatally. Lower amounts of Akt reduce protein production and metabolism of the lambs, which affects the health

of the lambs both immediately and later in life. There have been no direct studies on the effect of maternal nutrition on Akt synthesis.

As there was no change observed in the production of Akt in the muscle, studying the activation of Akt was the next target. Akt is activated by phosphorylation at its serine473 and threonine308 residues (Alessi et al., 1996). Ser473 lies in the hydrophobic motif and the Thr308 lies in the activation domain. Both are essential for the activation of Akt protein. A point mutation replacing these residues with alanine arrests cell proliferation, one of the main functions of active Akt (Alessi et al., 1996). We observed no effect of maternal nutrition on the phosphorylation at either of the two residues at birth. At three months of age, the lambs from ewes with a restricted diet showed a significant increase in phosphorylation at the serine residue. This suggests that in an attempt to compensate for the dampened metabolic reactions owing to suboptimal nutrient availability during fetal growth, the body activates Akt to bring the metabolism of the body to normalcy.

Poor maternal nutrition does not affect postnatal myogenesis via myostatin or follistatin

The role of myostatin in negatively regulating myogenesis has been extensively studied, however the effect of maternal nutrition on myostatin protein expression has been less explored. Research conducted by Liu et al. (2011) noted an increase in the myostatin gene expression in 8-month-old pigs when exposed to a low protein diet during fetal development. Hoffman et al. (2013) also observed similar results in newborn lambs from ewes provided with low energy diet during gestation. Our study showed no effect of maternal nutrition on the protein expression of lambs in the semitendinosus muscle.

As there was no change in myostatin protein expression, we studied the expression of a follistatin, which is a well-studied inhibitor of myostatin, hypothesizing that an increase in

follistatin could be a potential reason for the myostatin data that we obtained (Amthor et al., 2004). However, we did not observe a change in the expression of follistatin. The study performed by Hoffman et al. (2013) and our studies were on the same group of animals. The observation that the increase in mRNA expression of myostatin did not translate to similar change in the protein expression indicates that mechanisms like supercoiling of DNA, or binding of micro RNAs (miRNAs) to myostatin may prevent the translation of the gene to its protein (Valencia-Sanchez et al., 2006; Liu et al., 2011).

No inflammatory environment in the muscle of offspring exposed to poor maternal nutrition

Obesity is often accompanied by an inflammatory response (Hotamisligil et al., 1995; Weisberg et al., 2003; Xu et al., 2003). The best way to gauge an inflammatory response is to quantify the amount of proinflammatory cytokines at the site of inflammation. Our hypothesis was that the muscles of lambs from poorly nourished ewes, especially the obese ewes, would exhibit elevated levels of cytokines associated with inflammation in the muscle. Contrary to our hypothesis, there was no change in the expression of IL-6 or TNFα in the muscle of any of the lambs suggesting that poor maternal nutrition might not have induced a local inflammation in the muscle. However Barker et al. (1998), Boney et al. (2005) and Schmatz et al. (2010) report the induction of systemic inflammation in offspring from obese mothers at a very young age. An assessment of the inflammatory cytokines in the plasma would be our next strategy to understand whether maternal obesity promotes an inflammatory response in the fetus.

In conclusion, poor maternal nutrition has long-term effects on the health of the offspring. This is evidenced in our study of muscles from lambs of poorly nourished ewes that show retarded growth of muscle fibers, and also by an increased number of type IIb myofibers in

the semitendinosus muscle at birth. In an attempt to rectify the effects of programming during fetal life, there is also an increase in the phosphorylation of Akt protein at its Ser residue. Further studies need to be performed on fetal stages for targeting the major pathways that can be altered due to poor maternal nutrition. The study on pathways that are affected during fetal development can be pursued for postnatal studies. Transcriptome analysis is one of the emerging tools to identify the causes of many diseases that have not been explained by any other genetic or molecular studies (Woroniecka et al, 2011; Farkas et al., 2013; Zapata et al., 2013). An examination of the entire transcriptome by RNA next generation sequencing has revolutionized the understanding of complex disorders, and this could give an unbiased report of all the genes that are modified due to poor maternal nutrition during gestation. Following this, the whole proteome analysis can also be studied. Proteome analysis will help understand the proteins that undergo post-translational modifications, have elevated expression or are inhibited as a result of nutritional insult during gestation. Transcriptome and proteome analysis together would also be able to identify potential therapeutic targets in humans as well as livestock to improve the quality of life postnatally. Another area that requires intensive research is regulation of satellite cells in the muscle during poor nutrient conditions. Many studies have observed a decrease in the reservoir of satellite cells with age and with adverse nutritional status (Allbrook et al., 1971; Day et al., 2010). The satellite cells are the only source of postnatal muscle growth. The satellite cell growth and development could be studied in our sheep model for poor maternal nutrition. The pathways studied for the whole muscle could be analyzed in the satellite cells as well to determine the specific pathways affected in these postnatal precursors of muscle fibers. Once the affected pathways have been identified, in vitro studies can be performed to target the pathways

by designing analogs for the specific molecules affecting each pathway. The analogs should be designed to specifically target the muscle to prevent non-specific dysregulation of pathways.

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